Transcriptomic Analyses of Inner Ear Sensory Epithelia in Zebrafish

Transcriptomic Analyses of Sensory Epithelia in the Inner Ear of Zebrafish

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Abstract

Transcriptome analysis can provide crucial information that will help in understanding the genetic mechanisms that control differentiation, proliferation, senescence, metabolism, morphology, and function of a cell or tissue under normal and pathological conditions. Recently, zebrafish model is gaining increasing attention for the study of the development and function of the vertebrate inner ear. The aim of this study is to examine the differential gene expression in saccular, utricular, and lagenar maculae of zebrafish, which will help us to understand the molecular basis underlying functional differences among three otolith organs. In this study, sensory epithelia were carefully dissected out from the saccule, utricle and lagena of adult transgenic zebrafish (*Et(krt4:GFP)*^{sqet4}), their respective total RNAs were isolated and analyzed by RNA GeneChip microarray. We observed that there was differential expression of genes in the saccule, utricle and lagena. We uncovered hundreds of differentially expressed genes in the three otolith organs. Some of these differentially expressed genes are related to the otolith development and balance in zebrafish or mice, or related to the deafness in humans. However, some of the genes were conserved among all three otolith organs. Uniquely expressed genes accounted for <10% of all genes in either otolith organ. The present study provides a dataset that will help in identifying and exploring the roles of deafness and balance related genes. It will further help in validating the utilization of zebrafish as a model to study human auditory and vestibular disorders.

Key words: Zebrafish inner ear; Neurosensory epithelium; Transcriptome analysis; Hearing; Microarray

Introduction

Microarray technology is a revolutionary tool used to query samples for analyzing thousands of genes simultaneously (Kierzek et al., 2015; Jia et al., 2017; Chen et al., 2017). By using this technology, we can get huge amount of data including the information on the genes of interest and can get clues about the physiological function of genes (Serifi et al., 2016). Microarray is an ideal tool for biological and medical study (Streets et al., 2017; Song et al., 2017; Dittmann et al., 2017). The traditional methods of gene detection include enzyme digestion, Restriction Fragment Length Polymorphism (RFLP), and direct sequencing. These methods are not quantitative, time consuming and expensive, and more importantly, these methods are unable to detect multiple mutations in different genes at a given time.

Zebrafish has become an important model for studying human genetic diseases due to its easy maintenance, embryo transparency, *in vitro* fertilization, various mutant species, and has solid genetic study methods (Grati et al., 2015; Nicolson, 2005; Whitfield, 2002; Yariz et al., 2012). With the advent of new transgenic and imaging technologies that allow the analysis of phenotypes in astonishing detail, the zebrafish will remain at the forefront of hearing research for many years to come. CRISPR/Cas9 can be easily employed in zebrafish to verify the function of the gene by the reverse genetics technique (Hwang et al., 2013; Jao, Wente, & Chen, 2013; Zou et al., 2015); Shaw et al., 2017) . Therefore, reverse genetics as well as forward genetics techniques can be used to deduce the genetic developmental pathway in zebrafish. There is an 84% similarity between the genomes of zebrafish and humans, which makes the zebrafish an ideal model for studying genes implicated in human hearing loss (Howe et al., 2013). However, no information is available

regarding transcriptomes of zebrafish inner ear that limits our ability to extrapolate the findings to humans.

The inner ear of adult zebrafish has three otolith organs (the saccule, utricle and lagena) that are very similar in structure, each of which is composed of a cell-packed sensory epithelium coupled with an otolith (Platt, 1993). There are only the saccule and utricle in the otic vesicle during the first week post fertilization, and the lagena is formed after 7 days post-fertilization (Bang, Sewell, & Malicki, 2001; Haddon & Lewis, 1996). It has been demonstrated that in zebrafish the saccule is a major hearing organ and the utricle is a key balance organ (Bever & Fekete, 2002; Riley & Moorman, 2000). However, the function of lagena is still unknown. To understand the gene expression pattern of zebrafish inner ear, and to differentiate the genes expressed in hearing and balance sensory epithelia, we examined the transcriptomes of all three otolith organs using microarray analysis. We observed that some of the genes are conserved in three otolith organs whereas other genes are differentially expressed among these otolith organs. The identification of uniquely or differentially expressed genes can reveal the molecular basis underlying the unique physiological functions of otolith organs in zebrafish. This study provides a unique zebrafish transcriptome dataset that will be of immense use to understand the biological properties of the saccule, utricle and lagena, as well as will further validate the use of zebrafish model to understand the biology of the human auditory system.

Materials and Methods

Dissection and isolation of Zebrafish otolith organs.

Transgenic *Et(krt4:GFP)*^{sqet4} zebrafish (Gleason et al., 2009; Go, Bessarab, & Korzh, 2010; Lu & DeSmidt, 2013; Parinov, Kondrichin, Korzh, & Emelyanov, 2004; Zamora & Lu, 2013) at 2 years old were used for the experiments in this study. The animal care protocol for all procedures used

in this study was approved by the University of Miami Animal Care and Use Committee (IACUC) and complies with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. After zebrafish were anaesthetized with 0.01% buffered MS-222 solution, individual otolith organs such as saccules, utricles, and lagenas were carefully dissected out from the brain cavity as described by Liang and Burgess (Liang & Burgess, 2009). Under a dissecting fluorescence microscope (Zeiss SteReo Discovery V20), the otolith was removed from each otolith organ, and the nerve bundle attached to the sensory epithelium was peeled away with a pair of fine forceps. Finally, the membrane surrounding the neurosensory epithelium was trimmed off using a pair of Vannas spring scissors. Eight sensory epithelia from each otolith organ were collected.

RNA extraction and purification.

Total RNA, including small RNAs (more than 18 nt), from saccular, utricular and lagenar epithelia separately suspended in RNAlater were extracted and purified using the Qiagen miRNeasy Mini Kit. On-column DNase digestion was performed to further eliminate DNA contamination in the collected RNA. Quality and quantity of RNA were determined using an Agilent 2100 BioAnalyzer. The experiment was repeated twice for two separate biological replicates.

GeneChip Microarray.

An amount of 15-ng total RNA per sample was prepared using the Ovation Pico WTA System V2 (part#3302-12) and yielded cDNA product that was fragmented and labeled using the Encore Biotin Module according to the manufacturer's protocol. Affymetrix Zebrafish Gene 1.0 ST Array (part# 902007) were scanned using GeneChip Scanner 3000 7G system. The Affymetrix GeneChip

Command Console Software (AGCC) was used to perform background subtraction, normalization and assess quality control metrics before being passed on for analysis.

Data Analysis.

Data analysis followed a detailed procedure specific for zebrafish 1.0 ST array (Musso et al., 2015). In particular, raw CEL files were processed using the Oligo package (Carvalho & Irizarry, 2010) as part of the Bioconductor suite (www.bioconductor.org) in the R statistical framework (www.r-project.org). The background subtraction and normalization were performed using the Robust Multiarray Average (RMA) method implemented in the Oligo package. Boxplots of intensity values were compared for all chips before and after normalization to visualize the corresponding effects on mean and quartile values. Following normalization, probeset IDs were matched to corresponding transcript IDs. Specifically, the zebrafish 1.0 ST array NetAffx annotation file was downloaded in CSV format from the Affymetrix website (www.affymetrix.com), and transcript/gene IDs corresponding to given probe IDs were extracted. All transcript and gene IDs were mapped to corresponding Ensemble gene IDs. For Ensemble transcript IDs, corresponding Ensemble gene IDs were obtained using the BioMart community portal (Smedley et al., 2015). The Synergizer web application (Berriz & Roth, 2008) was used to convert gene IDs from other annotation frameworks to Ensemble gene IDs. Batch effects have been discovered by principle component analysis (PCA) plot and removed using function removeBatchEffect from Bioconductor package limma (Law et al., 2016; Koper et al., 2017). Differential expression analysis was tested under default parameters ($P < 0.05$, FDR < 0.05).

Measurement of gene expression in sensory tissues by RT-qPCR

Tissues from the inner ear were dissected as described above and RNA from Utricle, Saccule and Lagena was extracted using Quick-RNA microprep kit (Zymo Research), and further purified using RNA Clean & Concentrator kit (Zymo Research). cDNA was synthesized using Superscript III First-strand synthesis kit of RT-PCR (Life Technologies Inc.) from 1 µg of total RNA. The synthesized cDNA from individual tissues was used as template for RT-qPCR using gene specific primers. The RT-q PCR was performed using 1X Platinum SYBR Green qPCR SuperMix-UDG (with ROX reference dye at a final concentration of 50 nM) and 0.3 µM each of the forward and reverse primers. The gene expression was quantified using amplification and dissociation curves following $2^{-\Delta\Delta Ct}$ method, and values from Utricle was used for normalization to compare the gene expression in Saccule and Lagena.

Results and Discussion

In order to ensure the accuracy and quality of sensory epithelia that we dissected for this study, we used transgenic *Et(krt4:GFP)^{sqet4}* zebrafish to isolate the otolith organs. The hair cells of transgenic *Et(krt4:GFP)^{sqet4}* zebrafish expresses green fluorescent protein (GFP) (Gleason et al., 2009; Go et al., 2010; Grati et al., 2015; Lu & DeSmidt, 2013; Parinov et al., 2004; Zamora & Lu, 2013). The GFP fluorescent marker facilitates morphological observations of live hair cells and quantification of sensory epithelium of zebrafish inner ear (Supplementary Figure S1). The experiment was repeated twice to provide two biological replicates for the analysis. The RNA expression profile of three otolith organ sensory epithelia was determined by microarray analysis. Figure 1 shows the principle component analysis (PCA) plot after removing batch effects. Samples of the same otolith organs are clustered together, indicating the samples are separated by the biological variation of interest. About 86.9% (PC1, 59.1% plus PC2, 27.8%) of variance can be explained by the

biological variation of the three different types of otolith organs. As demonstrated in Figure 1, the saccule and utricle are located far apart from each other, with lagena lying in the middle of these two otolith organs.

To determine which genes are expressed in the saccule, utricle and lagena, we analyzed the transcripts in each otolith organ. Figures 2, 3 and 4 shows the expression levels of the top 200 genes in the saccule, utricle and lagena, respectively. For comparison, expression levels and abundance rankings for the same transcripts in all the three otolith organs are also illustrated. As shown in all three figures, the vast majority of the known genes and the uncharacterized transcripts that were found abundantly expressed in one otolith were also highly expressed in the other two otolith organs. Some of the genes were abundantly expressed in all the three otoliths organs including *runt-related transcription factor 1* (*runx1*), mitochondrial cytochrome oxidases including *mt-co1*, and *mt-co3* as well as mitochondrial encoded NADH dehydrogenases such as *mt-nd1-4*.

Next, we analyzed genes that are differentially expressed in the saccule, utricle and lagena, because these genes may underlie unique structures and functions of each otolith organ. We compared the expression levels of all the transcripts in the saccule with those of the utricle and lagena. Figure 5 illustrates the most differentially expressed genes in the saccule compared to the utricle (A) and lagena (B). We observed higher expression levels of *wnt11r*, *cep41*, *prox1a*, *rarra*, *sema3e*, *gdf10a*, *otol1a, ctgfa*, *nr2f1a*, and *vwa2* in the saccule. These genes have also been demonstrated to be expressed in the otic vesicle in zebrafish in previous studies that corroborates with their relevance in hearing function of saccule in zebrafish (Glasgow & Tomarev, 1998; Lee et al., 2012; Lu & DeSmidt, 2013; Pistocchi et al., 2008) Maier *et al*., 2014) (http://zfin.org/). The functions of *cep41*, *aldh1a2, otol1a*, *ctgfa*, and *nr2f1* (mouse orthologous genes) have been related to ear

morphology and otic capsule development in mouse models (http://www.informatics.jax.org/). *prox1a* is widely expressed in the mouse inner ear, especially in the cochlea (Dominguez-Frutos et al., 2009; Gray et al., 2004; Hartman, Hayashi, Nelson, Bermingham-McDonogh, & Reh, 2007; Hume, Bratt, & Oesterle, 2007). *Rara* has shown to be expressed in a wide variety of mouse ear compartments, such as in the vestibular component, the middle ear labyrinth, the organ of Corti, the limbus lamina spiralis, the spiral ligament, and the stria vascularis (Gray et al., 2004; Romand et al., 2002; Visel, Thaller, & Eichele, 2004). Knock-out mouse model for *rara* orthologue showed hearing abnormalities (Lufkin et al., 1993). *Sema3e, gdf10*, and *ctgf* expression has been observed in the cochlea, otic capsule, pharyngo-tympanic tube, and tubotympanic recess of mice (Diez-Roux et al., 2011). Knock-out mouse models for *ctgf* orthologue showed abnormalities in hearing and vestibular functions (Doherty, Kim, Hiller, Sulik, & Maeda, 2010; Ivkovic et al., 2003). Similar phenotypes were observed in a knock-out mouse model (Qiu et al., 1997) for the *nr2f1a* orthologue that has been shown to be expressed in the cochlea (Jonk et al., 1994). In a patient with CHARGE syndrome and carrying a de novo balanced translocation involving chromosomes 2 and 7, translocation breakpoints were mapped and *Sema3e* orthologue was identified within 200 kb of the breakpoint on 7q21.11 (Lalani et al., 2004; Martin, Sheldon, & Gorski, 2001). The chromosomal locations of human orthologues of *cep41*, *cbln4*, *wnt11*, *masp1*, *aldh1a2*, *pla2g4c*, *sema3e*, *c1qtnf5*, *entpd5a*, and *apcdd1l* have been associated with hereditary hearing loss in human patients (http://hereditaryhearingloss.org/). Figure 6 shows the differentially expressed genes in the utricle compared to the saccule (A) and lagena (B). Figure 7 shows the differentially expressed genes in the lagena compared to the saccule (A) and utricle (B). The comparisons show quite different gene expression profiles in three otolith organs, indicating different functional roles of these otolith organs in the zebrafish ear. The agreement of the differential gene expression patterns

between our study and previous studies validates the high purity of three otolith organs used for this study.

Next we analyzed the differential expression of 96 transcripts to investigate their potential structural and functional contributions in the auditory system. We observed the expression of *actn1, anxa6, arf1, argap17a, calm2a, calm2b, cdh23, dpysl2b, chd3, flnb, fscn2b, pls1, twf2b, ush1c, xirp2a, ywhaqa, and ywhaqb* genes (Figure 8). There are no studies available regarding the functional characterization and physiological relevance of these genes in the auditory system of zebrafish. However, some of these genes have been shown to be expressed in the mouse auditory system and implicated in maintaining the structure of stereocilia as well as in hearing. The expression of *calm2, cdh23, dpysl2b, flnb, pls1, twf2b, ush1c* orthologues have been observed in the mouse auditory system (Di Palma et al., 2001; Diez-Roux et al., 2011; Ficker, Powles, Warr, Pirvola, & Maconochie, 2004; Johnston et al., 2004; Kamata et al., 1998; Kamiya et al., 2014; Lelli, Asai, Forge, Holt, & Geleoc, 2009; Visel et al., 2004). *Pls1*-null mice have a moderate and progressive form of hearing loss across all frequencies and the stereocilia of inner hair cells in these mice were reduced in width and length starting at an earlier age (Taylor et al., 2015). Another gene *USH1C* was mapped to the *PDZ73* gene to chromosome 11p15.4-p15.1 by genomic sequence analysis and FISH (Scanlan et al., 1999). Mice with spontaneous hypomorphic mutations and knock-out for *USH1C* showed behavioral, hearing, and vestibular abnormalities (Grillet et al., 2009; Johnson et al., 2003; Lefevre et al., 2008; Tian et al., 2010). Similarly, several phenotypes such as age related hearing loss, degeneration of stereocilia, hair cell death, behavioral changes, loss of harmonin in apex of hair cells have been observed in mouse models generated by chemical induction, knock-outs, and spontaneous methods for *cdh23* gene (Bahloul et al., 2010; Di Palma et al., 2001; Han et al., 2014; Manji et al., 2011; Noben-Trauth, Zheng, Johnson, & Nishina, 1997;

Schwander et al., 2007; Zheng et al., 2005). In human studies, *USH1C* mutations were determined to be the cause of Usher syndrome type I in seven Acadians, one Pakistani and one Canadian homozygous for the Acadian alleles (Ouyang et al., 2003), in two siblings from a Caucasian British family with hearing loss diagnosed at 4 years of age and retinitis pigmentosa (Saihan et al., 2011), and in 12 patients from 8 Israeli families of Yemenite Jewish origin with retinitis pigmentosa and late-onset hearing loss (Khateb et al., 2012). In a Cuban family, two different *CDH23* mutations were identified (Bolz et al., 2001). Two nonsense and two frameshift mutations in the *CDH23* gene in 4 families with *USH1D*, (Bork et al., 2001), three novel mutations in 33 patients with type I Usher syndrome in whom *USH1B* and *USH1C* had been excluded (von Brederlow et al., 2002), a comprehensive catalog of 33 novel *CDH23* mutations with recessive nonsyndromic deafness or Usher syndrome type I (Astuto et al., 2002), 3 families with Usher syndrome type I in which affected members carrying mutations in both *CDH23* and *PCDH15* (Zheng et al., 2005) have been observed.

Clic4, slc17a8, tmc1, tmc2a, tmc2b, trpv4 have been demonstrated to be highly expressed in zebrafish in saccular, lagenar, utricular, crista and neuromast hair cells , which is in agreement with the findings of the present study (Figure 9) (Amato et al., 2012; Einhorn, Trapani, Liu, & Nicolson, 2012; Gabashvili, Sokolowski, Morton, & Giersch, 2007; R. Maeda et al., 2014; Mangos, Liu, & Drummond, 2007; Obholzer et al., 2008). In case of mice, *Clic5* has been shown to express in saccular and utricular maculae (Gagnon et al., 2006). Mice homozygous for a spontaneous mutation in *Clic5* exhibit head bobbing and circling behavior, inability to swim, and complete deafness by 7-8 months of age caused by dysmorphic stereocilia and progressive hair cell degeneration. *Slc17a8* is not expressed in the auditory system, however, studies on mouse models show changes in behavior, hearing, vestibular ear, and nervous system, (Gras et al., 2008;

Seal et al., 2008). *tmc1* expression has been shown in otic capsule, macula of utricle, urticle, and cochlea of mice by *in situ* hybridization (ISH) (Diez-Roux et al., 2011; Kawashima et al., 2011). Spontaneous mutation and knock-out of *Tmc1* in mouse models have shown hearing defects Knock-out mouse models for *tmc2* that is expressed in macula of utricle, urticle, cochlea of mice While mice with a targeted deletion of Tmc1 (Tmc1(Δ) mice) were deaf and those with a deletion of Tmc2 (Tmc2(Δ) mice) were phenotypically normal, Tmc1(Δ)Tmc2(Δ) mice had profound vestibular dysfunction, deafness, and structurally normal hair cells that lacked all mechanotransduction activity (Kawashima et al., 2011)).

In humans, a truncating mutation in the *CLIC5* were found to be the cause of autosomal recessive deafness-103 in two siblings of Turkish decent (Seco et al., 2016). By genomic sequence analysis, *tmc1* gene was mapped to the chromosome 9q13-q21 (Kurima et al., 2002). In a North American Caucasian family, the autosomal dominant nonsyndromic postlingual progressive sensorineural hearing loss was linked to the DFNA36 locus on chromosome 9q13-q21; Mutations in the *tmc1* gene in affected members of 10 Pakistani families with autosomal recessive DFNB7/11 were identified (Kitajiri, Makishima, Friedman, & Griffith, 2007) , seven Turkish families (Hilgert et al., 2008) and members of a large 6-generation Chinese family with DFNA36(Zhao et al., 2014). By genomic sequence analysis, the *tmc2* gene was mapped to chromosome 20p13 (Kurima et al., 2002) and *TRPV4* gene to chromosome 12q24.1 (Liedtke et al., 2000).

The information about the genes involved in regulating cell cycle can provide important clues about the development and maintenance of the zebrafish inner ear. The genes related to cell cycle in the saccule, utricle and lagena are shown in Figure 10. We observed the expression of *brca2, ccnb2, cdkn1ba, cdkn1bb, cdkn1ca, gadd45aa, mad2l1, mcm4, notch2,* and *pmp22b* in all the three otolith organs. The expression of *cdkn1b* has been seen in the cochlea, cochlear duct epithelium,

and organ of corti of mice (Chen, Johnson, Zoghbi, & Segil, 2002; Hartman et al., 2007; Nagahama et al., 2001), and *Notch2* in the mouse otocyst (Hamada et al., 1999). *Ccnb2* has been mapped to the chromosome 15q22.2. *Cdkn1c* has been associated with Beckwith-Wiedemann Syndrome (BWS) in nine unrelated Japanese patients and detected mutations in two BWS patients (Hatada et al., 1996). Lam *et al.* (1999)(Lam et al., 1999) sequenced the *Cdkn1c* gene in 70 patients with BWS. Fifty-four were sporadic with no evidence of uniparental disomy and 16 were familial from 7 kindreds. Novel germline *Cdkn1c* mutations were identified in 5 probands, 3 of 7 familial cases and 2 of 54 sporadic cases. *Cdkn1c* expression is reduced in patients with Beckwith-Wiedemann syndrome (BWS) with allele imbalance and its haploinsufficiency contributes to the BWS phenotype in patients with mosaic paternal isodisomy of chromosome 11 (E. M. Algar, Deeble, & Smith, 1999). Another study examined 32 patients with BWS for mutations affecting the *Cdkn1c* gene, including 7 cases of familial BWS (E. Algar, Brickell, Deeble, Amor, & Smith, 2000). Seven novel mutations in the *CDKN1C* gene have been identified in 8 of 50 patients with BWS (Romanelli et al., 2010). The *MAD2L1* gene has been mapped to 5q23-q31 by fluorescence *in situ* hybridization (L. Xu et al., 1997).

Figure 11 shows the expression of over 80 genes that have been implicated in human deafness or related to hearing in animal models. *col11a1a, col11ab, col11a2, col4a5, dfna5b, eya1, eya2, grhl2a, grhl2b, kcnq4, otofa, otofb, and slc26a5* are highly expressed in the zebrafish otic vesicle, hair cells, and semicircular canal (Albert et al., 2007; Baas, Malbouyres, Haftek-Terreau, Le Guellec, & Ruggiero, 2009; Blasiole et al., 2006; Chatterjee et al., 2015; Croushore et al., 2005; Fang, Adams, McMahan, Brown, & Oxford, 2010; Hoffman et al., 2010; Janicke, Renisch, & Hammerschmidt, 2010; Landgraf et al., 2010; Wang et al., 2015; Wu et al., 2014; Xiao & Baier, 2007; Yokoi et al., 2009)b). *col11a1* has been shown to express in the inner ear and otic capsule

of mice (Diez-Roux et al., 2011; Yoshioka et al., 1995) and spontaneous mutation mouse models show both auditory and vestibular abnormalities. Although *col11a2* expression has not been observed in the auditory system of mice, a knock-out model still seems to show vestibular abnormalities (Li et al., 2001). Abundant *Dfna5* expression has been observed in the cochlea and inner ear of mice (Y. Maeda, Fukushima, Kasai, Maeta, & Nishizaki, 2001) and *Dfna5* knock-out mice develop hearing abnormalities (Van Laer et al., 2005). *Eya1* is expressed in otocyst, otocyst epithelium, inner ear, otic capsule, and vestibule (Abdelhak et al., 1997; Lillevali, Matilainen, Karis, & Salminen, 2004; Ozaki et al., 2004). Spontaneous *Eya1* knock-out and targeted mouse models show hearing abnormalities (Ahmed et al., 2012; Johnson et al., 1999; P. X. Xu et al., 1999). *Grhl2* expression in mice has been observed in the inner ear, cochlea, middle ear, pharyngo-tympanic tube (Diez-Roux et al., 2011; Visel et al., 2004). Similarly, *kcnq4* expression has been observed in the utricle (Holt, Stauffer, Abraham, & Geleoc, 2007), and knock-out mouse models for this gene show hearing abnormalities (Kharkovets et al., 2006). *Otof* expression has shown to be expressed in the inner ear of mice (Schwander et al., 2007; Wilson et al., 2005). Otoferlin deficient mice (*Otof* $^{-/-}$) are profoundly deaf demonstrating complete abrogation of exocytosis in inner hair cells (IHCs) (Roux et al., 2006). *Slc26a5* expression has been observed in the cochlea of mice (Lelli et al., 2009) and radiation, targeted and knock-out models show hearing abnormalities (Gao et al., 2007; Liberman et al., 2002; Palos et al., 2008). The mutations in *SLC26A5* cause deafness in two Japanese siblings (Mutai et al., 2013), *col11a2* in American, Dutch, Iranian, Tunisian and Turkish families (Chakchouk et al., 2015), *DFNA5* in two Dutch families, two Chinese families, one Iranian family and a 328-member cohort study (Van Camp et al., 2002; Van Laer et al., 1997; Van Laer et al., 2008; Yu, Wylie-Sears, Boscolo, Mulliken, & Bischoff, 2004). In a large American family with an autosomal dominant form of progressive

nonsyndromic sensorineural hearing loss, a mutation in *GRHL2* was identified (Peters et al., 2002). Similarly, in affected members of a large 5-generation family, the *GRHL2* mutation caused deafness (Vona, Nanda, Neuner, Muller, & Haaf, 2013). A heterozygous mutation in *KCNQ4* has been identified in a large deaf family and it was concluded that *KCNQ4* related hearing loss is intrinsic to outer hair cells and *KCNQ4* forms heteromeric channels with *KCNQ3* (Kubisch et al., 1999). A study on three families from the Netherlands and Belgium (Van Camp et al., 2002) and 2 from Indonesia and the United States (Coucke et al., 1994) found missense mutations in *KCNQ4* gene. *KCNQ4* was also shown to be the cause of deafness in a 5-generation American family (Talebizadeh, Kelley, Askew, Beisel, & Smith, 1999), Dutch family (Van Hauwe et al., 2000), and 4-generation Spanish family (Mencia et al., 2008). Three Indian siblings with *OTOF* mutation suffered profound hearing loss (Yasunaga et al., 2000), and one out of four Druze families had a novel *OTOF* mutation (Adato, Raskin, Petit, & Bonne-Tamir, 2000). In one Cuban family, two Spanish families, and eight sporadic Spanish patients, an *OTOF* mutation was associated with nonsyndromic sensorineural hearing loss (Migliosi et al., 2002). The screening for the same *OTOF* mutation was extended to 289 additional unrelated families, finding 15 new cases, 9 of which were homozygous and 6 of which were heterozygous (Rodriguez-Ballesteros et al., 2003). Thus, the deafness genes which we identified in zebrafish have implications in human hearing loss validating the use of zebrafish models to identify and characterize human deafness genes.

To further confirm our microarray data, we performed real-time quantitative PCR. We used the top differentially expressed genes in three otolith organs from our microarray data. We computed the log2 fold difference between the expression of genes in saccule and lagena using utricle as the normalizer. As shown in Figure 12 we observed that the differentially expressed genes identified by the two techniques, RT-PCR and microarray, are highly consistent. These differentially

expressed genes may provide valuable information to understand different biological properties (such as structural and functional differences) of three otolith organs.

In summary, the present investigation is the first genome-wide transcriptome study that examines the gene expression profiles of the saccule, utricle and lagena of adult zebrafish. We observed that although the expression of some genes are conserved between three otolith organs, however there are few genes that are differentially expressed in the saccule, utricle and lagena. These differential expressed genes may impart unique structures and physiological functions to these otolith organs. This dataset provides a unique tool to the auditory and neuroscience community to explore the functional roles of these genes in future studies employing CRISPR/Cas9 genome editing.

Author Contributions

AAD, BZ, MG, DY, RM, QY, RM, DY, ZL, TR, KL, and XL designed and performed the experiments; ZL and XL supervised the study; QY, LY, RM, DY, ZL and XL contributed to data acquisition; QY, LY, RM, BZ, AKS, DY, ZL GV, and XL contributed to data analysis and wrote the manuscript.

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Figure Legends:

Figure 1: Principal component analysis (PCA) of microarray expression levels across three different otolith organs in zebrafish. The X-axis represents the greatest source of variance across all samples and the Y-axis represents the second-greatest. Each symbol represents the results of one microarray hybridization, as indicated in the legend. Red symbols represent RNA samples from the first batch and blue symbols represents RNA samples from the second batch. Six samples are well grouped by different otolith organs, indicating that there is less variance between samples of the same otolith organs than between two different batches. The two greatest principal components account for 86.9% (59.1% + 27.8%) of the variance.

Figure 2: Expression levels of top 200 genes in Saccule. The bars show the fluorescent density of each gene after normalization. Numbers in green, red, and blue signify the abundance rank of the genes in Saccule, Utricle and Lagena respectively. In this and all subsequent figures, ENSMUST identification numbers are truncated to show the last six digits.

Figure 3: Expression levels of top 200 genes in Utricle. The bars show the fluorescent density of each gene after normalization. Numbers in green, red, and blue signify the abundance rank of the genes in Saccule, Utricle and Lagena respectively.

Figure 4: Expression levels of top 200 genes in Lagena. The bars show the fluorescent density of each gene after normalization. Numbers in green, red, and blue signify the abundance rank of the genes in Saccule, Utricle and Lagena respectively.

Figure 5: Differentially expressed genes in Saccule. The numerical values represent the fold difference in expression in Saccule versus Utricle (A) or Saccule versus Lagena (B).

Figure 6: Differentially expressed genes in Utricle. The numerical values represent the fold difference in expression in Utricle versus Saccule (A) or Utricle versus Lagena (B).

Figure 7: Differentially expressed genes in Lagena. The numerical values represent the fold difference in expression in Lagena versus Saccule (A) or Lagena versus Utricle (B).

Figure 8: Expression level of genes related to stereocilia structure in Saccule, Utricle and Lagena.

Figure 9: Expression level of genes related to Ion channels in Saccule, Utricle and Lagena.

Figure 10 Expression level of genes related to cell cycle in Saccule, Utricle and Lagena.

Figure 11 Expression level of genes related to deafness in Saccule, Utricle and Lagena.

Figure 12: Expression levels of genes in Saccule and Lagena using quantitative reverse transcription PCR (RT-qPCR), values from Utricle were used for normalization.